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ROUSHAN ISLAM AND BEJOYSEKHAR DATTA

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Department of Botany,
University of Calcutta,
Kolkata 700 019, India

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Siderophore producing *Fusarium* isolates exhibit *in vitro* antagonism against plant pathogenic fungi

ROUSHAN ISLAM[*1] AND BEJOYSEKHAR DATTA[2]
[1]Department of Botany, Acharya Prafulla Chandra Roy Government College, Himachal Vihar, Matigara, Siliguri 734010, West Bengal
[2]Department of Botany, University of Kalyani, Kalyani 741235, West Bengal

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The genus *Fusarium* represents one of the important groups of filamentous fungi, abundant in soil as free-living saprophytes, comprising ofboth pathogenic and non-pathogenic species. In the present study, *Fusarium* species were isolated from soil samples collected at two different cultivated fields of Murshidabad district in West Bengal, India where no *Fusarium* diseases were reported previously. The isolates were characterized by the morphological studies. Siderophore production by the *Fusarium* isolates was tested using chrome azurol sulfonate reagent. Three *Fusarium* soil isolates were selected due to their high production of siderophore (400-600 nmole/ml). Isolate SF104 produced maximum siderophore. Hydroxamate nature of the siderophore was revealed following Czaky's assay. Non-pathogenic nature of the isolates was also confirmed by observing any/no inhibitory effect of the culture filtrate of the isolates on seed germination of gram and cucumber plants. Antagonistic activity of the *Fusarium* isolates was examined by dual culture technique against five plant pathogenic fungi *viz*., *Alternaria solani, Curvularia lunata*,*Fusarium oxysporum*, *Helminthosporium oryzae* and *Rhizoctonia solani*. All the *Fusarium* isolates were highly antagonistic to all the five fungal pathogens with percentage of inhibition varied from 60 to 90 %. Isolate SF104 showed maximum antagonism against *R. solani* inhibiting both mycelial growth and sclerotia formation. Thus, these *Fusarium* soil isolates have the potential to act as efficient biocontrol agents.

Key words: *Fusarium*, siderophore, hydroxamate, phytopathogens, antagonism

INTRODUCTION

Soil microorganisms like fungi have a particular important role in exploration of new approaches. One of the beneficial activities of the soil borne microorganisms is the production of siderophore(s). The word siderophore is defined as relatively low molecular weight, ferric ion specific chelating agent produced by bacteria and fungi growing under low iron stress condition. Iron is essential for the growth of almost all organisms. It is required in metabolic processes such as TCA cycle, electron transport chain, oxidative phosphorylation and photosynthesis. Being a component of cell, iron deficiency can cause growth inhibition, decrease in RNA/DNA synthesis, inhibition of sporulation and change in cell morphology. But in many environments, the amount of free iron is below 10^{-7} M. Under conditions of iron starvation, to compete effectively with hydroxyl ion for the ferric state of iron, microor-

*Corresponding author : rislammsd@gmail.com

ganisms synthesize iron chelators known as siderophores. The function of these compounds is to scavenge iron from the environment and to make it available to the microbial cell. Regulation of the siderophore production is based on the concentration of iron in the environment. So, siderophore production is shut off when iron is present at sufficient concentration and *vice versa.* The siderophores chelate iron in the extracellular environment and the resulting ferric siderophore complex is recognized by siderophore specific membrane receptors, enabling uptake of iron by the microbial cells . Although considerable structural variation exists among the several dozen siderophores chemically characterized at the present time, most can be classified as hydroxamates or catechols. Generally, fungi are stronger siderophores producers than bacteria and mostly produce hydroxamate type siderophores which are typically composed of three hydroxamate groups linked by peptide or ester bonds to form an octahedral complex.

Siderophore mediated suppression of soil borne plant diseases is one of the most studied mechanisms involved in biocontrol by fungi. Control of phytopathogens by biological means was environmentally advantageous in comparison to chemical control methods which had many risks on human health and environment (Parani and Saha, 2009). Biological control is thus being considered as an alternative way of reducing the use of chemicals in agriculture.

The present study on *Fusarium* spp. was concentrated on the screening of their ability to produce siderophore, siderophore typing and growth inhibition of five selected plant pathogenic fungi, under *in vitro* condition. The applications of purified siderophore as bacteriostatic or fugistatic agents in combination with other antimicrobial factors will certainly raise great interests in the fields of agriculture.

MATERIALS AND METHODS

Collection of soil samples

Soil samples at a depth of 6 cm were collected from agricultural fields of Bamnabad village (soil sample 1) and Lochanpur village (soil sample 2) located in Raninagar block 2 (near Indo-Bangladesh boarder region) of Murshidabad district in West Bengal, India. Both the agricultural fields were situated in the close vicinity of the Padma river and cultivated for various crops throughout the year where no *Fusarium* diseases were reported previously.

Isolation and characterization of Fusarium spp.

The fungi were isolated directly from the soil by dilution plate technique on potato sucrose agar (PSA) medium [composition (g/l): potato 200, sucrose 20, agar 20, pH 6] supplemented with pentachloronitrobenzene (0.1 %) and chloramphenicol (0.01 %) for selective growth of *Fusarium* spp. The plates were incubated at 28 °C for 5-7 days until visible sign of colony growth occurred. The fungal isolates were grown on Czapek's Dox agar (CDA) and synthetic nutrient agar (SNA) medium for their subsequent characterizations. The composition of CDA medium was (g/l) K_2HPO_4 1, $MgSO_4$, $7H_2O$ 0.5, KCl 0.5, $NaNO_3$ 2, sucrose 30, agar 20. The composition of SNA medium was (g/l) KH_2PO4 1, $MgSO_4$, $7H_2O$ 0.5, KCl 0.5, $NaNO_3$ 1, glucose 0.2, sucrose 0.2, agar 20. Reproductive structures of the isolates were also studied through microscopic observation.

Siderophore production test

Fusarium isolates were grown in Czapek's Dox broth (without addition of $FeSO_4$) at 28 °C for 30 days and the culture filtrate was assayed for detection siderophore using CAS reagent (Schwyn and Neilands, 1987) with desferal as standard. Chemical nature of the siderophore was determined by the method of Czaky (1948) and Arnow (1937) for hydroxamate and catecholate type, respectively.

Study of antagonism of Fusarium spp. *against phytopathogenic fungi*

Fusarium isolates were tested for their antagonistic properties against five fungal pathogens, *Alternaria solani, Curvularia lunata*, *Fusarium oxysporum*, *Helminthosporium oryzae* and *Rhizoctonia solani* by dual culture technique (Idris *et al*, 2007). Mycelial discs (5 mm) from each pathogen and antagonist were placed 3 cm apart on fresh PDA plates and the plates were kept at 28 °C for 7 days. The antagonism effect was determined by observing inhibition of mycelial growth of the patho-

gen and percentage of inhibition (I) was measured using the formula: I = (R – r)/R X 100; where r = radius of the pathogen colony opposite the antagonist colony and R = maximum radius of the pathogen colony in untreated (control) plate.

RESULTS AND DISCUSSION

Isolation and characterization of Fusarium spp.

The genus *Fusarium* represents one of the important groups of filamentous fungi, abundant in soil as free-living saprophytes, comprising of both pathogenic and non-pathogenic species. The genus *Fusarium* has received much attention due to its high degree of variability. Leslie and Summerell (2006) reported more than 70 species under the genus. The pathogenic species of *Fusarium* are responsible for destructive diseases on cereal grains, vascular wilts or root rots of many important vegetable, ornamental and field crops. There are reports to control of *Fusarium* wilt by using non-pathogenic species of *Fusarium*. The non-pathogenic isolates with potential biocontrol attributes can be utilized to suppress plant diseases.Out of seven *Fusarium* isolates recovered from the two soil samples, three isolates (SF0104, SF0203 and SF0204) were considered for the study regarding siderophore production. All the three isolates were fast growing species producing white compact mycelial colony. SF0104 and SF0203 produced orange and pink pigment, respectively but no pigment was produced by SF0204. Detailed of their reproductive structures is given in Table 1. Moreover, the isolates were non-pathogenic and did not inhibit seed germination of gram and cucumber plants.

Siderophore production by Fusarium spp.

Fungal siderophores are generally of the hydroxamate in nature which might be either of three types, ferrichrome, coprogen, fusarinine. *Fusarium roseum* strain ATCC 12822 produced malonichrome, a ferrichrome siderophore. *F. oxysporum* strain FGSC 9935 reported to produce three different ferrichrome type siderophores: ferricrocin, ferrichrome C, and malonichrome (Lopez-Berges *et al*., 2012). In this study we could obtain three *Fusarium* soil isolates positive for siderophore production (Table 2). The type of siderophore produced by these isolates was solely hydroxamate as has been revealed by Czaky assay. Among the soil isolates, SF0104 was found to be the best siderophore producer (600 nmole/ml). On the other hand, SF0204 and SF0203 produced siderophore 500 and 400 nmole/ml, respectively. A correlation between growth (mycelia dry weight) and siderophore production by the isolates was also established (Table 2). Highest mycelia dry weight was observed in SF0104 (0.268 g) which subsequently produced highest siderophore (600 nmole/ml). In another study, it was found that the *Fusarium* isolates SF0204 and SF0203 also produced substantial amount of IAA and promoted the growth of both gram and cucumber seedlings considerably (Islam and Datta, 2015).

Antagonism of Fusarium spp.

Fast growth is important character for a potential antagonist used in biological control of plant pathogens. It suggests strong competition for nutrients and space, which is very useful for the inhibition and possible elimination of pathogens. All the three *Fusarium* antagonists were fast growing in nature (Table 1). Three types of interaction between antagonist and plant pathogens have been recognized: antibiosis, competition for nutrients and hyperparasitism (Woo *et al*, 2006). In dual culture assay, all the three *Fusarium* isolates were found to inhibit the growth of the five phytopathogens to a reasonable extent with percentage of inhibition (I) varied from 60 to 90 % (Table 3). Maximum antagonism was observed against *Rhizoctonia solani* by all the three isolates inhibiting both mycelial growth and sclerotia formation (I varied from 85 - 90 %) and minimum inhibition was observed against *Fusarium oxysporum* (I varied from 60 - 71 %). Although the antagonism mechanism of the *Fusarium* isolates was not identified in this study, it might be suggested that their antagonism must have involved the production of siderophore as well as other antifungal metabolites. Siderophore directly stimulates the biosynthesis of other antimicrobial compounds by increasing the bioavailability of iron and other minerals which would suppress the growth of pathogenic microorganisms. After the additional incubation period, the pathogenic mycelia did not cover the surface of the tested soil isolates, which indicates that the antagonism was very strong. Interestingly, the extreme siderophore producer *i.e.* SF0104 was found to be the best antagonist against all the five pathogens. Dalal *et al,* (2014) reported that *Fusarium* isolate JDF12 produced siderophore and

Table 1 : Growth characteristics and sporulation of the *Fusarium* soil isolates on CDA medium

Isolate no.	Source	Colony on CDA medium	Sporulation
SF0104	Bamnabad (Soil sample 1)	White, circular, 83 mm in diameter, with faint orange pigment	Macroconidia wide, straight to slightly curved, medium sized (15 -20 µm X 3.12-3.75 µm) with 2 -3 septa; apical cell blunt and rounded, basal cell foot - shaped with a notched or a rounded end. Microconidia sparse, ellipsoid, fusiform, short sized (6.25 -10 µm X 1.25-2.5 µm) with 0-1 septa; monophilide. Chlamydospores abundant on CDA and SNA media, intercalary, terminal, single, in pair, globose (10-12.5 µm in diameter), smooth and rough walled.
SF0203	Lochanpur (Soil sample 2)	White, circular, compact, 83 mm in diameter, with faint pink pigment	Macroconidia wide, straight to slightly curved, medium sized (16.25-26.25 µm X 3.12-3.75 µm) with 1-3 septa; apical cell blunt and rounded, basal cell foot - shaped with a notched or a rounded end. Microconidia sparse, ellipsoid, fusiform, medium sized (6.25-10 µm X 1.25 -2.5 µm) with 0 -1 septa; monophilide. Chlamydospores abundant on CDA and SNA media, intercalary, terminal, single, in pair, globose (10 -12.5 µm in diameter), smooth and rough walled
SF0204	Lochanpur (Soil sample 2)	White, circular, more compact, 82 mm in diameter, with no pigment	Macroconidia wide, straight to slightly curved, medium sized (16.25 -26.25 µm X 3.12-3.75 µm) with 2-4 septa; apical cell blunt and rounded, basal cell foot - shaped with a notched or a rounded end. Microconidia sparse, ellipsoid, fusiform, medium sized (6.25 -10 µ X 1.25-2.5 µ) with 0-1 septa; monophilide. Chlamydospores abundant on CDA and SNA media, intercalary, terminal, single, in pair, globose (10-12.5 µ in diameter), smooth and rough walled

exhibited antifungal activity against a number of phyto-pathogens. Similarly, *Pseudomonas fluorescens*produced siderophores and antifungal metabolites which were involved in the control of plant pathogenic fungi. Patil *et al,* (2015) also reported that *Bacillus subtilis* capable of producing hydroxamate type of siderophore acted as a biocontrol agent due to their efficacy to inhibit the fungal phytopathogens. It was suggested that microbial siderophore stimulate plant growth directly by increasing the availability of iron in the soil surrounding the roots or indirectly by competitively inhibiting the growth of plant pathogens with less efficient iron uptake system.

Table 2 : Growth and Siderophore production by *Fusarium* isolates in Czapek's Dox broth medium

Fusarium isolates	Mycelial dry weight (g)	Amount of siderophore produced (nmole/ml)
SF0104	0.268	600
SF0203	0.200	400
SF0204	0.255	500

Soil microorganisms are known to improve the plant growth directly through nutrient mobilization, production of plant hormones and indirectly through suppression of plant pathogens. Biological methods offer an excellent alternate strategy for effective control of various diseases and plant growth promotional activity. These findings clearly demonstrated the relative efficacy of *Fusarium* soil species in production of metabolites like siderophore which may indirectly involved in pathogen suppression. Thus the present studies serve as a good indication of siderophore producing *Fusarium* spp. as potential biocontrol agents. In addition, ecological advantages in the synthesis of fungal siderophore will also encourage the use of these *Fusarium* isolates as bioinoculants. A further research in this field can bring about solutions for other related problems faced in the fields of agriculture.

Table 3 : Bioefficacy of *Fusarium* soil isolates against fungal pathogens by dual culture assay

Plant pathogenic fungi	Percentage of inhibition by *Fusarium* soil isolates		
	SF0104	SF0203	SF0204
Alternaria solani	80	77	80
Curvularia lunata	71	75	77
Fusarium oxysporum	60	71	62
Helminthosporium oryzae	77	77	80
Rhizoctonia solani	90	88	85

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